University of Maine

Maine Agricultural Experiment Station

ORONO

BULLETIN No. 178. APRIL 1910.

AN *ENDOMYCES* FROM APPLE

This bulletin contains a description of a new species of *Endomyces* which was isolated from decaying fruit of the apple in Maine, the results of inoculating apples with the fungus, cultural characters, and a comparison of this species with other species of Endomyces and with species of certain other closely related genera.

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BULLETIN No. 178.

A NEW SPECIES OF ENDOMYCES FROM DECAYING APPLE.

CHARLES E. LEWIS.

In October 1908, the fungus which is described in this paper was discovered by the writer in connection with a study of apple decay fungi at this Station. In examining material from an apple which showed a small injured place which was overgrown by fungus mycelium and had a rather dry appearance, the spores of several fungi including species of *Alternaria, Cladosporium,* and *Fusarium* were found. Associated with these were a large number of almost spherical brown bodies which were for the most part 11-14 microns in diameter, each of which was found to contain 4 spores. Dilution plate cultures were made, using prune agar as a culture medium. Eight fungi developed in these plates and among them was one which produced conidia in very much the same manner which has been described by Brefeld (1) for *Ascoidea rubescens* Brefeld. The conidia were formed in such large numbers that they became piled up on the agar to such an extent as to give a powdery appearance. There was almost no development of aerial mycelium.

When the cultures were examined, 10 days after they were made, it was found that a large number of small bodies resembling the spore sacs or asci of an *Endomyces* had developed. These sacs were borne on short branches of the hyphae as shown in Figures 60, 61, and 65. Some of the sacs had already formed spores and in each case, so far as determined at that time, 4 spores were found. Later study has shown that there are usually a considerable number of the bodies which do not form spores and that there may be occasionally an ascus which forms only 2 spores. Cases in which only 2 spores are formed are very exceptional and are very rarely seen. No case has ever been observed in which more than 4 spores were formed. When the ascospores are mature they are brown in color. They vary in size according to the size of the ascus but in asci which

measure 11 microns in diameter the spores are about 4 x 5.5 microns. The ascospore has a thick wall with usually a number of thickened places on the wall. The asci or sacs which do not produce spores vary in size and appearance. In some cases they are of normal size and appear hyaline and seem to be lacking in contents. In other cases they are smaller, being 7-8 microns in diameter and contain a number of refractive bodies which are probably food material. Some of the smaller sacs in which no spores develop bear some resemblance to chlamydospores but they do not germinate when sown in hanging drop cultures.

The fact that the conidia and the spore sacs belong to the same fungus was determined by finding cases in which the branch bearing conidia was attached to the same hypha which was producing spore sacs. It was also determined by the fact that colonies which developed from a single spore began to produce conidia in 1 to 3 days and later the spore sacs began to develop so that in 6 to 10 days large numbers were found.

Since this fungus was isolated from a decaying apple, it seemed desirable to determine whether it could cause decay of apples upon inoculation. A number of apples were inoculated November 4, 1908, from pure cultures. A slow decay took place and after 10 days the decayed regions at the points of inoculation were each about 1.5 cm. in diameter. Some of the decayed tissue was removed from one of the apples and was teased out and examined. Large numbers of the spore sacs were found occupying the spaces between the cells. Plate cultures were made by taking out some of the decaying tissue from points inside one of the decaying apples with a sterilized scalpel and transferring to petri dishes containing prune agar. Pure cultures of the fungus with which the apple was inoculated were secured in each case showing that this fungus was responsible for the decay. One month after the time of inoculation, the fungus was reisolated in pure culture from an apple which was about one-half decayed. In the summer and fall of 1909, inoculations were made to determine whether this fungus could attack green apples and it was found that while in some cases it grew to a slight extent in the injured tissue at the point of inoculation in no case did it cause decay of unripe apples. It does not seem probable that this fungus will become of much importance as a cause of apple decay since it causes only a slow decay of ripe fruit.

The chief interest in its study lies in the fact that it belongs to a family of fungi no representative of which has been reported from America so far as the writer is able to determine. The character of producing spores in sacs or asci on short branches of the mycelium places it in the family *Endomycetaceae* and the fact that the 4 spored ascus is formed from a single branch and not from a fusion of two branches places it in the genus *Endomyces,* according to the classification of Schröter (12). The fungus under consideration, however, differs from the described species of *Endomyces* in certain morphological and cultural characters.

Endomyces decipiens (Tulasne) Rees, which ocurs as a parasite on *Armillaria mellea* Vahl, has asci which are 12-13 x 17 microns. This species produces chlamydospores which are 10-12 x 15-17 microns, and the mycelium breaks down to form oidia. *Endomyces Magnusii* Ludwig was described by Ludwig and was later studied by both Brefeld (2) and Hanson (6). It occurs in fermenting sap of the oak in Germany. The cells of the mycelium of this species separate very readily to form oidia. The asci do not develop in culture under ordinary conditions but Brefeld secured them by growing the fungus so that the mycelium was deeply buried in gelatin. The asci are large, being 25 x 40-45 microns. *Endomyces scytonematum* Zukal produces asci which are 17-18 x 25-26 microns. Each ascus contains 8 spores. *Endomyces meliolincola* Rehm has asci which are 45 microns in diameter. *Endomyces coprophilus* Massee and Salm. has asci which are 18-25 x 20-30 microns and contain 4-8 spores.

Endomyces parasiticus Fayod occurs as a parasite on *Tricholoma rutilans.* The hyphae are 2-3 microns in diameter, the asci are somewhat pear shaped, are 12 microns in length and 6-7 microns across at the broad end. This species differs from *Endomyces decipiens* and *Endomyces Magnusii* in producing conidia which are cut off from the end of a slender conidiophore and in the fact that the mycelium does not break down to form oidia.

The fungus which was isolated from apple, when grown upon a number of culture media, produces hyaline one-celled conidia after the manner shown in Fig. 66. The conidia vary somewhat in size, being 3-3.5 x 6-10 microns but for the most part mature conidia are about 3 x 8 microns. The hyphae vary in thickness from 3 to 6 microns. The asci are as a rule almost spherical,

are 11-14 microns in diameter and are borne on branches which vary considerably in length. Some asci are found which are less than 11 microns in diameter and some are found which are not spherical being somewhat pear shaped. In some cases also rather abnormal development of the spore sacs takes place as shown in Fig. 70, but by far the most common method of production of asci is for a number of single asci to develop on short branches from a hypha, as shown in Figures 60, 61, 62 and 65.

In order to determine the extent of variation in growth of the fungus and the effect upon its reproduction, it has been grown upon a rather large number of culture media.

Cultural Characters.

When the conidia are placed in a hanging drop of prune decoction, potato broth, or beef extract broth plus 2 per cent dextrose, at room temperature, they germinate readily. The conidia become considerably swollen and within 4-5 hours a number of them will begin to put out germ tubes. As a rule the conidium puts out only one germ tube but in some cases two are produced from opposite sides of the spore. In some cases, even in the hanging drop, the germ tube may grow out to form a branched mycelium on which short conidiophores bearing the conidia are borne as shown in Fig. 66. In other cases, the germ tube may grow out and begin to bud off conidia from the end within 16-18 hours. Some conidia are formed in very irregular ways as shown in Fig. 67. The conidia did not germinate in hanging drops of neutral beef extract broth.

The ascospores germinate, in some cases at least, while still in the ascus. Material from a culture 3 weeks old growing on bean agar and in which there were a large number of asci which contained spores was thoroughly washed to remove conidia and then teased apart and enough material to contain several asci in each case was placed in each of 5 hanging drops of prune decotion. After 16 hours, examination showed that in a number of asci one or more of the spores had germinated. Here, as in the case of germinating conidia, some of the germ tubes grow to form a branched mycelium and in some other cases conidia are formed on the end of the germ tube within 18 to 24 hours after the spores are sown as shown in Figure 69.

The characters of the growth upon a number of culture media will be given the temperature being about 20° C. in each case. The notes are based on observations of 2 or more tubes in every case, and with the agars on plate cultures as well.

Prune agar. When conidia are sown in plates of prune agar they germinate readily and by the end of 48 hours a much branched mycelium is formed with numerous conidiophores which bear large numbers of conidia. For good growth the medium must be acid. Prune agar was made alkaline to —2.5 Fuller's scale with NaOH, neutral, and acid to +2.5, +10, and +20 with HCl. There was no growth in the alkaline medium and very little in the neutral while the growth in the acid medium was good, that in +10 and +20 being equally good. The relation of the growth of the fungus to acids will be discussed later in this paper.

Prune decoction + 12. The growth in a decoction made by cooking 6 good sized prunes in 1000 c. c. of distilled water is very good. The mycelium develops rapidly in the bottom of the tube and after about 48 hours a film has developed over the surface of the liquid. The spore sacs or asci develop well in this medium and many are found which have formed spores at the end of 10 days. There is a good development of the mycelium and there is no tendency to break down to form oidia in the liquid medium as is the case with *Endomyces Magnusii.*

Potato agar +6. At 42 hours conidia had been produced in large numbers. After 5 days a few developing spore sacs were observed and when the cultures were 9 days old large numbers of spore sacs were seen, in some of which the spores had developed.

Potato broth. In neutral or alkaline potato broth there was no growth but when a drop of lactic acid was added to each tube before inoculation the fungus grew readily and at about the same rate as in prune decoction. Large numbers of asci were found 9 days after the tubes were inoculated.

Bean agar. The growth here is practically the same as on potato agar.

Bean pods. In bean pod tubes the growth is mostly confined to the liquid and to that part of the pod just above the liquid.

Vegetable plugs. On sterilized potato, beet, turnip, and car-

rot cylinders in tubes the growth is good. In 40 hours the entire slant of the plug is covered by the fungus and conidia are being formed. The conidia are formed in such abundance as to become piled up in considerable masses giving a wet slimy appearance. After conidia are formed, they germinate in some cases, producing short germ tubes on which conidia are formed in the irregular manner described as occurring in hanging drops. The vegetable plugs seem to be much more favorable to the development of conidia than asci, as the asci are not found in very large numbers.

Apple wood. On sterilized apple twigs in tubes the growth is good. There is considerable mycelium in the liquid and a thick slimy covering on the upper ends of the pieces of wood at the end of one week. Both conidia and asci are produced in large numbers.

Beef extract agar. It has been noted above that the spores did not germinate in hanging drops of neutral beef extract broth. On neutral beef extract agar no growth took place but when the agar was made acid to +20 with hydrochloric, lactic, or acetic acid the fungus grew to a slight extent.

Beef extract gelatin. On this gelatin, when made acid with lactic acid, the growth is about the same as on beef extract agar. No liquefaction of gelatin but there was very little growth.

Beef extract gelatin + dextrose. The fungus grows very well on this medium and causes some liquefaction of the gelatin at the end of one week. After one month the gelatin in the tubes was liquefied to a depth of 1.5 cm.

Sugar agars. Agars made by adding 1 per cent of dextrose, saccharose, lactose or mannite to beef extract agar. When these sugar agars were neutral, no growth took place, but when they were made acid the fungus grew on all of them. The growth on lactose and mannite is poor but on dextrose and saccharose the growth is good. The fungus produces a rather thick, whitish mass composed largely of conidia but does not form many asci.

Sugar broths. The same 4 sugars which were used in agars were also added to beef extract broth. One, 2, 5 and 10 per cent of dextrose were tried and gave about equally good growth. With the other sugars 2 per cent was added to the broth in each case. These broths were made acid to +15 with hydro-

chloric acid. The relative growth with the sugar broths is the same as with the sugar agars. Dextrose and saccharose give much better growth than lactose and mannite. Few asci develop in these broths in comparison with the number that develop on some other media.

Dunham's solution. Small amount of growth in this medium and in the same medium plus lactic acid.

Dunham's solution + sugars. When the 4 sugars used with agar and beef broth were added to Dunham's solution which had been made acid with lactic acid they gave about the same relative growth as with the agars and beef broth.

Glycerine agar. Five per cent glycerine added to beef extract agar and made acid with lactic acid. The growth is thick and somewhat wrinkled when the cultures are 5 days old. When a small piece was removed with a needle, it was found to be rather gelatinous and hard to tease apart. When examined with the microscope this material showed a very great number of asci. This medium seemed to be more favorable to development of asci than any of the other media which were used.

Rice. The fungus does not grow very well on rice sterilized in distilled water but it forms a considerable number of asci.

Corn. Enough crushed yellow dent corn was placed in each tube so that when distilled water was added to soak it up it made about 10 c. c. The fungus grew very well on this medium and produced both conidia and asci.

Milk. Fresh milk from which the cream had been separated was sterilized in the Arnold sterilizer. The fungus did not grow well in this medium but when a drop of lactic acid was added to each tube before inoculation, good growth took place.

Relation of Growth to Acids.

It has already been noted in several places in connection with the growth on various media that this fungus requires an acid medium for its growth. A considerable amount of work has been done and data obtained on this subject. The fungus has been grown in liquid media to which definite amounts of different acids have been added. For the most part, 2 per cent. dextrose broth has been used because it was found that this made a favorable medium. An attempt has been made to determine

three things: (1) The extent to which the growth of the fungus in a medium changes the acidity of that medium; (2) the relative effect of different acids on growth; (3) the amount of a given acid which it is necessary to add to prevent growth.

Three inorganic acids,—hydrochloric, sulphuric and nitric,—and 7 organic acids,—lactic, acetic, citric, tartaric, oxalic, malic, and formic,—have been used. Normal solutions or solutions of twice the normal strength in some cases have been used in making up the medium to a given acidity. The acid was added to the dextrose broth in most cases before sterilizing but in the case of some of the volatile acids, the acid was added cold after the tubes had been sterilized and the tubes were then incubated for 3 days to prove sterility before inoculation. The cultures were made in test tubes each containing 10 c. c. of the culture medium. The acidity of the media is expressed in each case in terms of Fuller's scale.

To determine what change takes place in the acidity of culture media due to the growth of the fungus, a large number of determinations were made. In a considerable number of cases uninoculated tubes were kept as checks and titrated on the same day as the inoculated ones. In determining the acidity, the entire contents of the tube were poured into a porcelain dish and the tube was rinsed with part of the 50 c. c. of distilled water which was added. The dish was heated over a flame until the contents boiled for about one minute and then the titration was made.

It was found that in the case of 2 per cent. lactose broth or of 2 per cent. mannite broth neither of which is a favorable medium for the growth of the fungus, there was little or no change in the acidity after 18 days. In 5 per cent glycerine broth, the acidity increased from +12 to +18 at the end of 11 days. In 2 per cent dextrose broth, the increase in acidity varied from one to two per cent as shown in the table given below. In 5 per cent and in 10 per cent dextrose broth the acidity increased from +20 to +53 in the first and from +15 to +52 in the second at the end of three weeks. The table which follows shows that the acidity of the two per cent dextrose broth in which the fungus had grown for 2 or 3 weeks did not change to any extent by allowing the fungus to continue its growth for 2 weeks longer.

Acid used.	Acidity.	Inoculated.	Titrated.	Acidity.	Titrated.	Acidity
Hydrochloric	+10	March 20, 1909	April 12, 1909	+27.5		
"	+20	" 26, 1909	" 13, 1909	+39	April 27, 1909	+40
"	+30	" 26, 1909	" 13, 1909	+51	" 27, 1909	+48
"	+40	" 26, 1909	" 13, 1909	+50	" 27, 1909	+55
Lactic	+20	" 26, 1909			" 27, 1909	+41
"	+30	" 26, 1909	" 12, 1909	+49	" 27, 1909	
"	+40	" 26, 1909	"		" 27, 1909	+58
"	+60	" 26, 1909	" 12, 1909	+78	" 27, 1909	+77
"	+80	" 26, 1909	" 12, 1909	+95	" 27, 1909	+95
"	+90	" 26, 1909	" 26, 1909	+100	" 27, 1909	+100
"	+100	" 26, 1909	" 26, 1909	+112	" 27, 1909	+110
"	+120	" 29, 1909			" 27, 1909	+145
"	+150	" 29, 1909			" 27, 1909	+180
"	+220	" 29, 1909			" 27, 1909	+245
"	+280	" 29, 1909			" 27, 1909	+311

There is considerable difference in the amount of different acids which can be added to 2 per cent dextrose broth before the growth of the fungus is checked or entirely prevented. With hydrochloric, sulphuric and nitric acids there is good growth up to and including +60 but no growth at +80. Of the organic acids used, it requires a smaller amount of formic acid than of any of the others to prevent growth. There was fairly good growth at +20 and at +30, very little at +40 and none at +50. Malic, lactic, citric, and tartaric acids give good growth to high acidities. Very little difference can be noted with these 4 acids up to and including +300. Malic acid was only tested to +350 but gave good growth at that acidity. Lactic acid gives a little poorer growth at +350 than at +300, at +400 there is very little growth and at +450 it can scarcely be detected. Citric acid gave good growth up to +500 and was not tested further. Tartaric acid gives good growth up to +200, above +200 growth takes place slowly but there is a small amount of growth up to and including +600. With oxalic and acetic acids the growth increases slowly in comparison with that in citric acid for example. The limits of growth with oxalic acid is between +100 and +150. With acetic acid there is very little growth at +200 and none at +250.

Fermentation Tubes.

The *Endomyces* from apple has been grown in fermentation tubes which were filled with 2 per cent dextrose, saccharose, lactose, and mannite broths, and with 5 per cent glycerine broth. In dextrose, saccharose, and glycerine broths the fungus grew well in the open ends of the tubes but did not grow in the closed ends. In lactose and mannite broths, the growth was poor and was confined to the open ends of the tubes. No gas was produced in any of these fermentation tubes.

Relation of Temperature to Growth.

The growth of the fungus on a number of culture media at 20 degrees C. has been given under the head of cultural characters. In order to determine the effect of lower temperatures, 2 tubes each of prune decoction, bean agar, potato agar, potato, beet, carrot and turnip cylinders in each case were inoculated and kept at temperatures of 15°-16° C., 12°-13° C., 8° C. and at 5° C. At 15°-16° C. and at 12°-13° C., there was a slow growth but not so good in either case as at 20° C. At 8° C. and at 5° C., there was no growth which could be noted at the end of one week while in check tubes at 20° C., the fungus had spread over the entire surface of the slants. When the tubes which had been kept at 5° C., and at 8° C. were placed at a temperature of 20° C. growth took place readily.

Thermal Death Point.

Cultures of the fungus two days old in thin walled test tubes each containing 10 c. c. of 2 per cent dextrose broth made acid to +10 were used. A method of heating the cultures very similar to that described by Smith (14) was used. Tubes were heated for 10 minutes at each degree from 46° C. to 53° C. Transfers were made to other tubes of 2 per cent dextrose broth. There was good growth from all tubes up to 50° C. At 51° C. and at 52° C., the growth came up very slowly and at first it seemed that the thermal death point was between 50° C. and 51° C. but since growth took place at 51° C. and at 52° C. after a few days and no growth took place from tubes at 53° C. it seems that some place between 52° C. and 53° C. should be regarded at the thermal death point.

Growth from Old Cultures.

In order to determine the length of time which the fungus would retain its vitality in cultures, transfers were made from

time to time from prune agar and prune decoction cultures in both of which the fungus produces both conidia and ascospores. These cultures were kept at the temperature of the laboratory. It was found that growth took place readily when transfers were made from prune agar cultures up to 5 months old provided the agar had not become dried out. In no case was growth obtained from agar tubes in which the agar was dry. Growth took place when transfers were made from prune decoction cultures 6 months old. At that time, the liquid was almost all evaporated and the tests were not carried further.

Endomyces Magnusii.

A culture of this fungus was secured from the Association Internationale des Botanistes and has been grown upon a number of the same culture media under the same conditions for comparison. This species has been studied by Ludwig (10), Brefeld (1), and Hansen (6), all of whom grew the fungus in culture on a number of media. Their accounts agree for the most part. A branched mycelium is produced, the cells of which separate very readily to form oidia. Brefeld found that when the mycelium was grown in such a way that it was buried by the culture medium it did not break down so readily to form oidia and asci were formed. Hansen did not find the asci in any of his cultures, but his description of the mycelium and oidia agrees with those of Brefeld and Ludwig.

In this study, *Endomyces Magnusii* has been grown on the following culture media: Prune agar, potato agar, bean agar, prune decoction, bean pods, potato, turnip, carrot and beet cylinders, sterilized oak wood, 1 per cent dextrose, saccharose, lactose, and mannite agars, 2 per cent broths of the same 4 sugars, 2 per cent dextrose gelatine, 5 per cent glycerine broth, 5 per cent glycerine agar, and in both alkaline and acid milk. The fungus grew well in all of these media except in mannite, lactose, and glycerine agars and broths and alkaline milk. When the milk was made acid, the growth was good. In all of the media which were used, the fungus goes through the same stages. First, there is a development of mycelium which very soon breaks down to form oidia. After a little time, the oidia become quite thick walled and in some cases almost spherical. In none of these cultures have asci been found although they have been searched for repeatedly.

Attempts have been made to produce the asci by sowing the oidia in petri dishes in 5 c. c. of agar or gelatin and then after this was solidified adding enough gelatin to almost fill the dish. In this way a considerable amount of buried mycelium was produced in some cases but no asci. It seems that the asci must require very special cultural conditions for their development. The readiness with which the cells of the mycelium separate to form oidia is illustrated by the behavior in hanging drop cultures. When the rounded, thick walled spores were sown in hanging drops of prune decoction they germinated readily. After 18 hours, nearly all had pushed out germ tubes, and when the germ tube reached a length of about 3 times the diameter of the spore a cross wall was formed. After only a few cells were formed, they began to separate to form oidia. At the end of 48 hours the drops were filled with the one celled rounded oidia.

It is of considerable interest to compare the cultural characters of *Endomyces Magnusii* and of the *Endomyces* from apple. It has been seen that while their characters are very different each one retains its characters even when grown under a wide range of conditions and does not vary to any great extent. For example *Endomyces Magnusii* forms oidia in large numbers in all the culture media used either solid or liquid and does not form asci readily in cultures while the *Endomyces* from apple never forms oidia under any of the conditions which have been tested and produces asci readily in most of the culture media.

Another interesting point of difference is in their growth in fermentation tubes. *Endomyces Magnusii* does not form gas in 2 per cent lactose, and 2 per cent mannite broth but it does form gas in considerable quantity in 2 per cent dextrose and in 2 per cent saccharose broth. In dextrose broth at room temperature gas began to appear on the fifth day and increased rapidly in amount until the eighth day when the gas occupied about 80 per cent of the closed arm in each case. Most of the gas formed was carbon dioxide. In saccharose broth the amount of gas was less, being about 50 per cent of the closed arm.

In connection with the statement of the fact that *Endomyces Magnusii* ferments dextrose and saccharose with the formation of gas, attention should be called to the conclusions of Ludwig, Hansen and Brefeld in regard to fermentation by this fungus.

Ludwig discovered the fungus in fermenting sap of the oak and he also found a yeast-like form associated with it which he regarded as belonging to the *Endomyces* and he therefore regarded *Endomyces Magnusii* as the cause of the fermentation. Hansen (6) made a careful study in which by means of gelatin plates he secured cultures of the *Endomyces* from single oidia. He also secured pure cultures of the yeast form of Ludwig from single cells. He found the two forms to be entirely distinct as no yeast form developed in his cultures of *Endomyces Magnusii*. He described the yeast as a new species, *Saccharomyces Ludwigii*. Hansen grew both of these organisms in a considerable number of liquid media including solutions of sugars, extracts of a number of common fruits, and beer wort. He found that either one growing in pure culture caused fermentation of dextrose and saccharose but that neither caused fermentation of lactose. Hansen did not find the asci in his study of *Endomyces Magnusii* and he regarded it as doubtful whether the asci described by Ludwig belonged to the same fungus as the oidia and mycelium. In a later paper, Ludwig (11) stated that he had found asci on mycelium which was also forming oidia and as has been stated Brefeld secured the asci in culture under special conditions. Brefeld makes the statement in his account of *Endomyces Magnusii* that it does not cause fermentation of fermentable liquids but he does not give an account of the work by which this was determined and so it is impossible to know what liquids were tested. My work confirms the account given by Hansen (6) that *Endomyces Magnusii* causes fermentation of dextrose and saccharose with formation of gas, but it does not ferment lactose.

Another place in which *Endomyces Magnusii* and the *Endomyces* from apple differ is in their effect upon gelatin when grown in dextrose gelatin. *Endomyces Magnusii* does not liquefy gelatin while it has been shown earlier in this paper that the *Endomyces* from apple does cause considerable liquefaction.

In relation of growth to acids, the two fungi agree rather closely. With most of the acids used the limits of growth were found to be about the same, but there were some exceptions. In oxalic acid there was no growth of *Endomyces Magnusii* at +80 and in tartaric acid there was no growth at +400 and above. In lactic acid, however, this species grew a little better at the high acidities than the *Endomyces* from apple. Neither

of these species will grow in an alkaline medium. When grown in a medium containing sugar, *Endomyces Magnusii* does not cause as great an increase in acidity as is brought about by the *Endomyces* from apple.

Cytological.

Considerable work has been done in an attempt to study the *Endomyces* from apple from a cytological standpoint. The fact that it grows readily and produces asci and spores in abundance on a large number of culture media would seem to make it a favorable object for study, but the difficulties of securing good fixation and the small size of the nuclei have made it impossible to determine many of the important points which it is desirable to have cleared up in regard to the behavior of the nucleus and the method of spore formation in this group of fungi. A few things have been determined, however, which are of some interest.

Material for study was taken from actively growing cultures on prune agar and in prune decoction and also from apples which had been inoculated with the fungus and which were decaying. Material from such decaying apples showed large numbers of spore sacs and this seemed to be a very favorable place for the development of the ascospores. A number of fixing fluids were tried including Flemming's of different strengths, chrom-acetic acid, picric acid, picro-formol, and absolute alcohol. Some of the material from agar cultures and from decaying apples was imbedded in paraffin and sections were cut 5 and 8 microns in thickness. Sections were stained with Flemming's triple stain and with iron haemotoxylin. Some good preparations for determining certain points were prepared by teasing out material which had been fixed, and staining on the slide with iron haemotoxylin. The method followed in doing this was to prepare the slide with egg albumen fixative just as for fastening sections to slide, then material which had been run down from higher grades of alcohol to 30 per cent alcohol or water, was teased out on the slide and the alcohol or water was allowed to evaporate, care being taken that the slide did not become entirely dry. When almost dry the slide was placed in absolute alcohol which coagulated the albumen and fastened the material to the slide. Such material was stained with iron haemotoxylin. Some of the best preparations were from material grown in

prune decoction and fixed in picro-formol and in absolute alcohol.

The conidia of this fungus are uninucleate. When a conidium germinates, it becomes very much swollen and usually puts out a single germ tube. The nucleus moves over to the side of the conidium from which the germ tube grows but does not move out much into the germ tube. When the germ tube has grown out to some length the nucleus divides and one of the daughter nuclei moves out into the germ tube which then divides by a cross wall giving two cells each of which contains a single nucleus. By further growth and division, a branched mycelium consisting of many cells is formed. Each cell is uninucleate. This differs from what Miss Stoppel (15) found in *Eremascus fertilis* in which the number of nuclei in the cells varies from one to 15, and in which 6 to 8 nuclei are found in the germ tube.

The ascus develops from a single branch from the mycelium and not from a fusion of two branches as is the case in *Eremascus*. One or more asci may develop from a single cell, Fig. 65. There is no fusion of nuclei in the ascus and none has been observed in the mycelium preceding the formation of the ascus. The nucleus does not move out into the developing ascus until the outer end of the branch has rounded out and has attained some size. The writer has observed a considerable number of cases in which a single nucleus had moved only part way into the ascus when the material was fixed. A number of cases have been observed in which it appeared that the nucleus divides at the opening of the branch from the cell of the mycelium, one nucleus going into the young developing ascus and the other remaining in the cell. It would seem that such a division would be necessary in the case of cells which produce more than one ascus. Two cases in which division is taking place at this point are shown in Fig. 70. It has not been possible to determine whether the nuclear divisions are mitotic or amitotic. In some cases, there is somewhat the appearance that would be given by the threads of a low form of spindle connecting the chromatin which is to form the two nuclei or the same appearance might be given by the separating of two nuclei formed by direct division. The nuclei are small but the greatest difficulty is that no method of fixation which I have tried brings out the nuclear figures with sufficient clearness to enable one to determine with

certainty exactly what takes place in the dividing nucleus. The same difficulty has been encountered by a large number of investigators who have studied the yeasts and other low forms of Ascomycetes and hence there is much confusion in the accounts given by different writers.

The young ascus becomes almost spherical in most cases and enlarges to the size of the mature ascus before the nucleus divides. After the single nucleus has passed into the rounded part, this portion is cut off by a cross wall. The young ascus contains granular cytoplasm which surrounds a rather large central vacuole. The nucleus as a rule occupies a position in that part of the ascus which is opposite the stalk. The nucleus, in material which is well fixed, shows very much the same structure which as been described for the nucleus of *Saccharomyces* by Guillermond (4). There is a nuclear membrane, a small amount of chromatin, and a small spherical body which appears to be a nucleolus. This body stains red with the safranin of the triple stain. Unless material is well fixed and carefully stained it is impossible to distinguish the nucleolus from the chromatin and there appears to be a single dense mass inside the nuclear membrane.

With regard to the method of nuclear division in the ascus, little could be determined. In the first division, figures have been observed which might be interpreted as mitotic divisions with the chromatin which is to form the daughter nuclei still held together by the spindle, but the same appearance might be given by the separation of the two nuclei after an amitotic division.

Four nuclei are produced by a second division and these become the nuclei of the 4 ascospores. On account of the extremely small size of the nuclei and of the spores when first formed, it has not been possible to determine the exact method by which the spores are formed. It has been found, however, that the young ascospores are surrounded by an epiplasm as is the case in typical Ascomycetes. The young spores increase in size until when mature they almost fill the ascus. The membrane of the spore becomes thickened to a rather heavy wall. The spore contains a single nucleus.

The cytoplasm of the developing ascus seems to be of different composition from that of the mycelium and conidia. If one treats material consisting of young asci, mycelium, and conidia

with iodine in potassium iodide, the mycelium, conidia and the very young asci are not stained, but asci which have begun to swell out into the spherical form and those which are older are stained brown. The ascospores are also stained brown by the iodine. Schiöning has observed the same in the case of *Saccharomycopsis capsularis* Schiöning.

Systematic.

As was stated in an earlier part of this paper, if we classify this fungus according to the classification given by Schröter (12) it would be placed in the genus *Endomyces,* and it has also been shown that this species differs from any of the described species of that genus. There would be another possibility in its classification and that is that it might belong among the filamentous forms of the *Saccharomycetaceae.* The classification of the Saccharomycetaceae has been discussed by Hansen (7) and by Guillermond (4). Miss Stoppel (15) also gives briefly the characters of the genera and also the characters of *Endomyces* in her paper on *Eremascus fertilis.*

The relationship between the Saccharomycetaceae and the Endomycetaceae seems very close. Such a form as *Saccharomycopsis capsularis,* for example, which has a well developed mycelium, and in which cells of the mycelium develop into asci bears a very close relation to an *Endomyces* in which the asci are formed on short branches from cells of the mycelium. *Saccharomycopsis* however, produces yeast like cells which multiply by budding, Schiöning (13) in his study found that it was possible to cause this fungus to develop in that way with almost no production of mycelium in certain culture media and that, on the other hand, in certain other culture media there was a good development of mycelium and not much of the yeast form. None of the species of *Endomyces* develop typical yeast like forms in culture. In *Endomyces decipiens* and *Endomyces Magnusii* the cells of the mycelium separate as oidia but there is no yeast like budding. In the fungus from apple described in this paper, conidia are produced on short conidiophores, and in some cases in liquid media, the conidia develop from short germ tubes as shown in Fig. 67. There is, however, no typical yeast like budding as the writer understands that term.

The Endomycetaceae cannot be separated from the Saccharomycetaceae on the basis of fermentation because in both fam-

ilies there are species which cause fermentation of certain sugars and other species which do not cause fermentation. *Endomyces Magnusii* and *Endomyces fibuliger* Lindner (9) cause fermentation.

On the basis of a sexual fusion of cells or of nuclei before the formation of endospores there is no reason for placing the Saccharomycetaceae in the Hemiascomycetes and the Endomycetaceae in the Euascomycetes because in both families there are species in which the ascus develops from such a fusion and other species in which the ascus develops without such fusion. Guillermond (5) gives a good discussion of this point with regard to the Saccharomycetaceae and shows that in some of them two vegetative cells fuse before the ascus is formed, in others there is no such fusion but the ascospores fuse in pairs upon germination ,and in some others as *Saccharomycopsis,* there is neither a fusion of cells before the formation of the ascus nor a fusion of the spores upon germination. In the Endomycetaceae, according to Schröter, there is included one genus, *Eremascus,* in which there is a fusion of cells before the ascus develops. In *Endomyces Magnusii,* there are some cases in which there is a fusion of cells before the ascus develops and other cases in which the ascus develops from a single branch from a cell of the mycelium. Whether there is a fusion of nuclei in this species has not been determined. In the other species of *Endomyces* the ascus develops from a single branch but it has not been determined whether there is a fusion of nuclei before the ascospores develop.

In the fungus which has been described in this paper, the ascus develops from a single branch from a cell of the mycelium and a single nucleus passes into the young ascus. There is no fusion of germ tubes at the time of germination. We have here then a fungus which corresponds with those yeasts in which there is no sexual fusion of cells either before or after formation of ascospores. So far as the development of mycelium or the formation of ascospores is concerned there would seem to be little basis for placing a fungus like *Saccharomycopsis capsularis* in the Hemiascomycetes and the fungus described in this paper in the Euascomycetes. However, the fact that this fungus does not reproduce by typical yeast like budding would prevent its being classified in the Saccharomycetaceae. It has seemed best to the writer, therefore, to classify it as a new species of the genus *Endomyces.*

Whether the spore sacs of the yeasts or of *Endomyces* should be regarded as true asci has been questioned. Brefeld regards the spore sacs in yeasts as sporangia and he considers such forms as *Endomyces* to be more highly developed than the yeasts because the number of spores in the spore sac has become more definitely fixed than in the yeasts. There are, however, certain species among the yeasts in which the number of spores seems to be as definite as in *Endomyces*.

Harper (8) has shown that the spore formation in asci differs from that in sporangia and that the young ascospores are cut out of the cytoplasm in such a way that they are surrounded by epiplasm while this is not the case in sporangia. Guillermond (5) has shown that the young ascospores in certain yeasts are surrounded by epiplasm, and Miss Stoppel (15) has shown that the same is true in *Eremascus fertilis*. In the *Endomyces* described below the writer has found the same condition. It is desirable that further cytological study should be made upon species of the Hemiascomycetes and upon the lower forms of the Euascomycetes as it seems probable that such study will lead to a clearer understanding of the relationship of these forms to each other and to the higher Ascomycetes.

Description of Species.

Endomyces mali n. sp. Branched mycelium with cross walls develops in a large number of culture media, conidia averaging 3 x 8 microns formed on short conidiophores or on the ends of short germ tubes, no typical yeast like budding, asci 11-14 microns in diameter, usually formed singly on short side branches of the mycelium without fusion of cells or nuclei, ascospores almost spherical but slightly elongated 4.5 x 5.5 microns, thickened places on walls, brown when mature. Fungus grows well in large number of culture media, in liquid media, as prune decoction, a pellicle is formed in one to 2 days composed of mycelium and conidia, mycelium in liquid as well as at surface, asci produced in 5-8 days both at surface and in liquid. Cultures take on a brownish color after ascospores are formed. No fermentation with formation of CO_2 in dextrose, saccharose, lactose, mannite or glycerine broth. Requires acid culture media for growth.

Found in decaying fruit of apple, Orono, Maine, causes small amount of decay of ripe apples.

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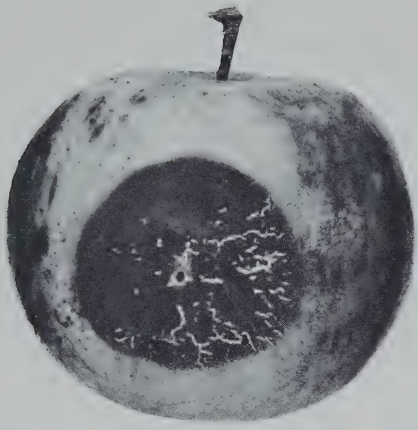

FIG. 58. Photograph of apple 2 weeks after inoculation with *Endomyces mali.*

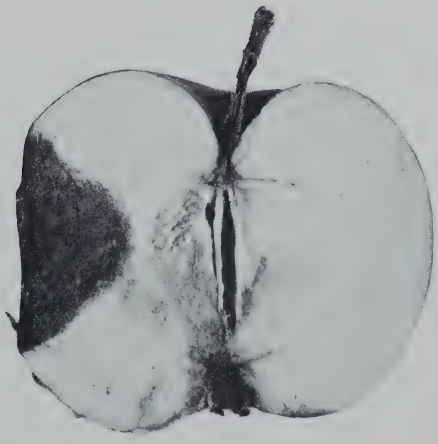

FIG. 59. Photograph of the same apple cut in two to show extent of the decay.

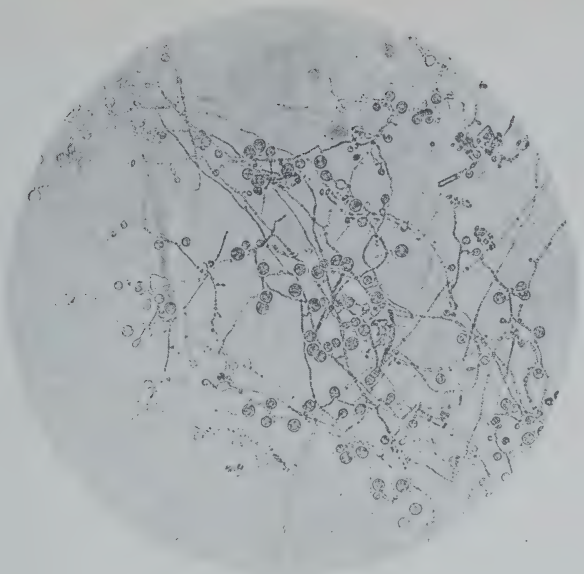

FIG. 60. Photo-micrograph of mycelium and young spore sacs of *Endomyces mali*. x 180.

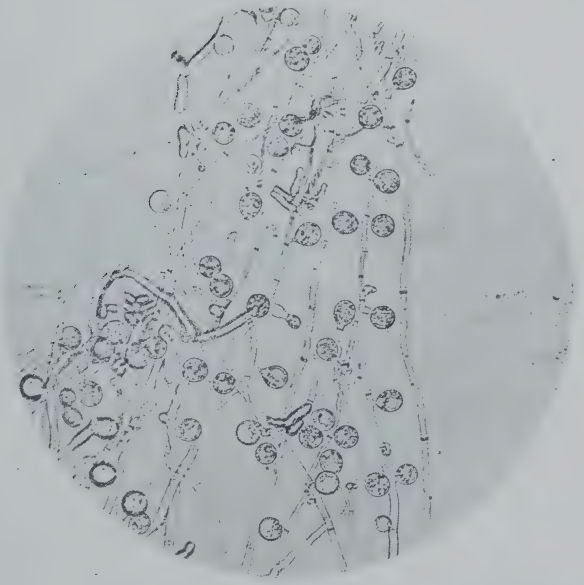

FIG. 61. Spore sacs and mycelium. x 350.

FIG. 62. Spore sacs more highly magnified. x about 800.

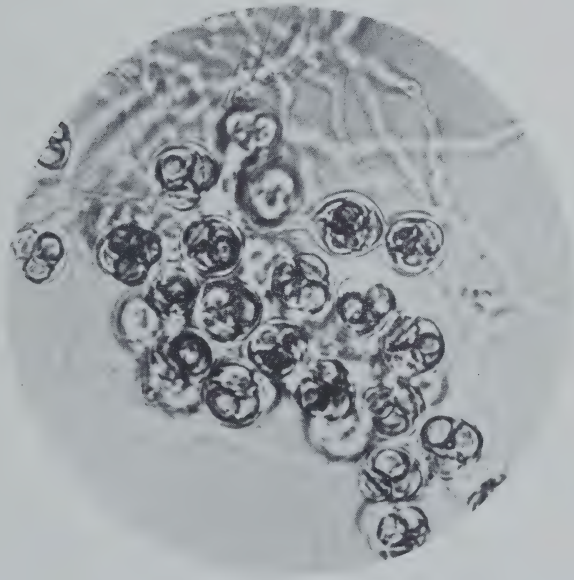

FIG. 63 Spore sacs which contain spores. x about 800.

FIG. 64. Mycelium from a single conidium after 42 hours in bean agar. x 350.

FIG. 65. Drawing of young spore sacs from unstained material. Nuclei cannot be seen here, a large vacuole in each ascus. x 480.

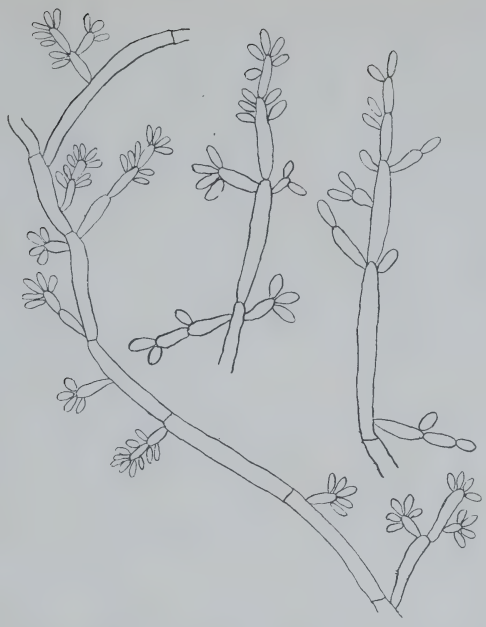

FIG. 66. Typical manner of bearing conidia on agar.

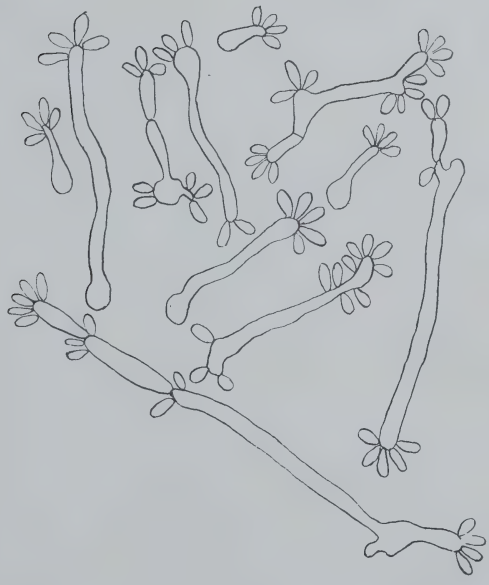

FIG. 67. Conidia formed on short germ tubes in hanging drop, prune decoction, after 18 hours. x 480.

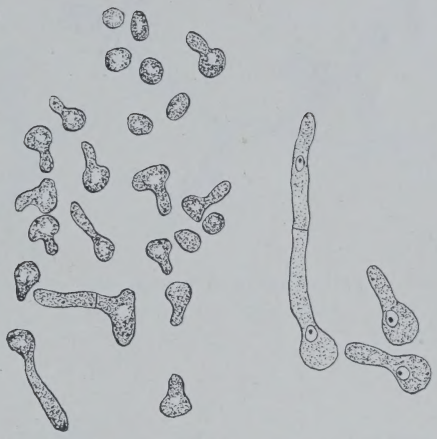

FIG. 68. Conidia germinating in hanging drop, prune decoction. At the left unstained, at the right stained to show nuclei.

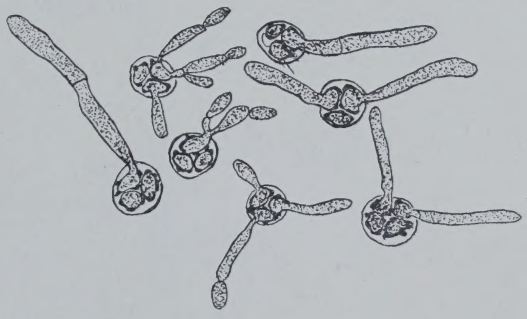

FIG. 69. Germinating ascospores.

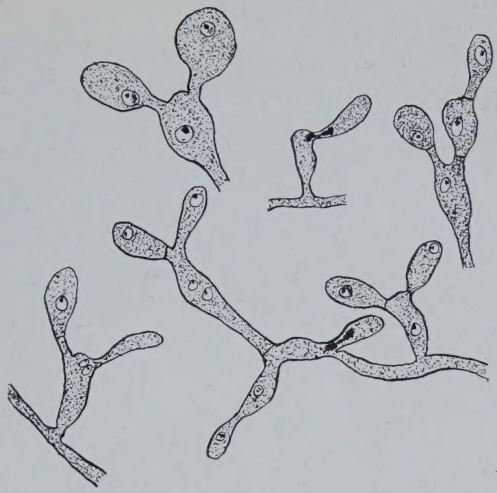

FIG. 70. Young spore sacs formed in irregular ways. Stained to show nuclei. x 800.

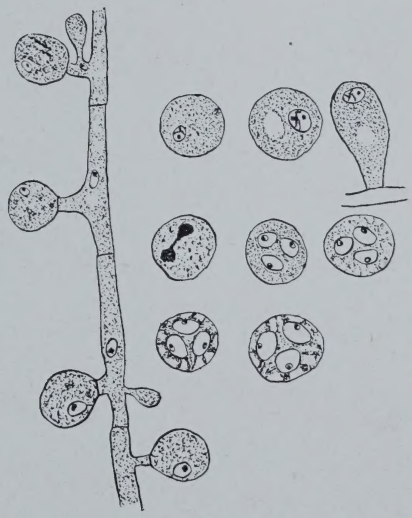

FIG. 71. At the left, young spore sacs 4 of which contain single nuclei, the nucleus has not moved into the younger ones; at the right above, 3 young asci showing typical nuclei, below, asci with young spores surrounded by epiplasm.